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Phospholipid acyl chain and phospholipase dynamics during cold acclimation of winter wheat

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Abbreviations: ESI-MS, electrospray ionizing mass spectrometry; LPC, lysophosphatidyl choline; PL, phospholipid; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D;

Abstract

Phospholipid (PL) composition is known to change in plants exposed to cold temperature. The dynamics of PL acyl chain pairs and genes encoding phospholipase enzymes were studied in winter wheat (*Triticum aestivum* L.) during cold acclimation. Mass spectrometry was used to characterize PL dynamics, and quantitative real-time PCR was used to characterize phospholipase gene mRNA transcript dynamics during cold acclimation. The proportion of PLs with mismatched acyl chains decreased concomitantly with an increase in total PLs during the first week of cold exposure. Proportions of mismatched acyl chains then increased, while total PLs varied little. Numbers of mRNA transcripts of phospholipase (PL)D, PLC, and PLA_2 increased in response to cold, and remained at elevated levels throughout a four-week period. Lysophosphatidylcholine (LPC) increased as much as 14-fold over the five-week period and increased significantly less in a less cold tolerant cultivar than more tolerant cultivars. It appeared newly-synthesized PLs with equal-length acyl chains form a part of the initial response to cold temperature; they are then modified to contain near-initial levels of mismatched acyl chains during acclimation. LPC is a highly active signal molecule and PLA_2 , PLC, and PLD are involved in generation of phospholipid-based signaling molecules; hence, it appeared PL signaling is involved in initial and continuing responses to cold temperature.

Changes in phospholipids in wheat plants responding to cold temperature have been investigated many times from a structural viewpoint. It repeatedly has been shown that the total phospholipid content increased during cold acclimation of wheat plants (De La Roche et al., 1972; De Silva et al., 1975; Horvath et al., 1980; Izzo et al., 1984; Willemot and Pelletier, 1980), and cultured cells (Sopin and Gavrilova, 1987). Horvath et al. (1980) proposed a predictive regression equation relating increased leaf phospholipid accumulation and acquired cold tolerance. Phospholipid *de novo* synthesis is not required for cold hardening (Willemot, 1975), and initial increases in phospholipid content were followed by decreases after three weeks (spring wheat) or five weeks (winter wheat) of cold exposure (De Silva et al., 1975). Very little difference was found in phospholipid concentrations from plants grown at 2°C compared to plants grown to a morphologically equivalent state, but over a shorter time, at 24°C (De La Roche and Andrews, 1973). Cold temperature appeared to have little effect on the phospholipid profiles. It has been suggested that alterations in concentration of specific phospholipids play a role in cold acclimation. Horvath et al. (1979) observed an inverse relationship between survival and the loss of phosphatidylcholine by conversion to the corresponding phosphatidic acid in field-grown wheat plants. A similar conversion of phosphatidylcholine to phosphatidic acid was reported by De La Roche and Andrews (1973) in plants grown at 2°C and morphologically equivalent plants grown at 24°C, again suggesting these phospholipid dynamics are a function of plant phenology rather than cold acclimation.

The proportion of unsaturated fatty acids, particularly linolenic acid (18 carbons, three double bonds [18:3]), increased during cold acclimation at the expense of less saturated forms (De La Roche et al., 1972, 1975; Skoczowski et al., 1994; Sopin and Trunova, 1991; Willemot and Pelletier, 1980; Willemot et al., 1977a, 1977b). However, it has been demonstrated that the accumulation of linolenic acid *per se* is not required for the development of freezing tolerance (De La Roche, 1979). Szalai et al. (2001) studied winter wheat, spring wheat, and

chromosome substitution lines incorporating winter wheat chromosome 5A, 5D, or 7A into the spring wheat background and concluded that the overall level of acyl chain fatty acid unsaturation was not related to cold tolerance. However, they reported that the rate of loss of *trans*-Δ^3-hexadecenoic acid, presumably due to its conversion to other fatty acids, was strongly correlated with cold tolerance. It also has been shown that the phospholipid composition of chloroplast thylakoid membranes changes during cold exposure, but the changes were not related to cold tolerance (Vigh et al., 1985).

Each of these studies examined the dynamics of the phospholipids defined by the head group and/or the fatty acid composition of the acyl side chains. However, we are aware of only one recent study where the composition of the acyl side chains was considered as a unit. Using electrospray ionizing mass spectrometry to analyze phospholipids extracted from *Arabidopsis*, Welti et al. (2002) characterized the acyl side chains as a combined group. For example, a 36:2 value would represent a particular pair of acyl chains containing a total of 36 carbons with two double bonds, probably indicating a pair of 18:1 acyl chains. The composition of the acyl chain pair of a phospholipid may play a crucial role in the behavior of the membrane or components embedded in the membrane. For example, the temperature optimum of membrane-bound ATPase varied significantly depending on the acyl chain composition of the surrounding phospholipids (Pitotti et al., 1980; Volmer and Veltel, 1985). Physical stability of a membrane protein was shown to be extremely dependent on the surrounding acyl chains (Maneri and Low, 1988), indicating a crucial role of the acyl chain pair in the stability of associated membrane components. Numerous nonphospholipid structures, including integral proteins, peripheral proteins, glycoproteins, glycolipids, and lipoproteins may be found in the membrane; hence, there is potential for the phospholipid acyl chain composition to influence the behavior of several membrane components.

The stability of the membrane structure itself is influenced by the composition of the pair of acyl chains. For example, phospholipids with mismatched acyl chains had increased average magnitudes of adhesion energy of the phospholipid bilayer (Fang et al., 2003), and acyl chain mismatches drastically altered elasticity (Ali et al., 1998) and the fluid dynamics of membranes following thermal perturbation (de Almeida et al., 2002). These phenomena probably are influenced by the very specific manner in which the acyl chains of different lengths assemble into the bilayer (Xu et al., 1987).

In plants, fatty acid chains are synthesized and elongated with pairs of carbon atoms (Somerville et al., 2000), and it has been reported many times that winter wheat fatty acids are comprised of about 20% 16-carbon and about 80% 18-carbon chains, with other chain lengths comprising minor fractions (Chirkova et al., 1981; De La Roche et al., 1975; De Silva et al., 1975; Skoczowski et al., 1994; Sopin and Gavrilova, 1987; Szalai et al., 2001; Willemot et al., 1977a). Odd-numbered carbon chains rarely have been reported in wheat. Therefore, acyl groups defined as, for example, 34:1, almost certainly indicate mismatched acyl chains (18-carbon paired with 16-carbon chains) because 17-carbon acyl chains comprise, at best, a small fraction of the fatty acids in wheat plants (Chirkova et al., 1981). Given these observations, we believe it is highly likely that, for example, 34-carbon acyl chain groups represent phospholipids with mismatched side chains, while 36-carbon acyl chain groups represent phospholipids with two 18-carbon (matched) side chains.

In addition to being essential structural components of cell membranes, it now is known that phospholipids play a much broader role; they can be signal molecules or signal precursors, or they may act as essential cofactors for membrane enzymes (Meijer and Munnik, 2003; Munnik et al., 1998). Typically, phospholipid-signaling systems are grouped according to the phospholipases that initiate the formation of the messenger molecules. Phospholipase D (PLD) releases the head group from phosphatidic acid; phospholipase C cleaves the

head group and the sn-3 phosphate from diacylglycerol (DAG), which usually is rapidly phosphorylated by DAG-kinase (Lundberg and Sommarin, 1992) to form phosphatidic acid. Phospholipase A_1 (PLA_1) cleaves the fatty acid residue from the sn-1 position and PLA_2 cleaves the fatty acid residue from the sn-2 position of the phospholipids (Meijer and Munnik, 2003). The products of PLA_1, A_2, C, or D activity have been implicated in signaling cascades (Laxalt and Munnik, 2002; Meijer and Munnik, 2003; Wang et al., 2000; Wang, 2002), which have been shown to regulate stress tolerance partly through modulation of stress-responsive gene expression (Zhu, 2002). Hence, phospholipid metabolism plays a crucial role in both cell signaling and structural development.

The studies implicating these phospholipase-mediated signaling pathways generally have focused on rapid response to various stress factors. Acclimation to cold temperature can be considered as a long-term response to stress with a complex series of metabolic outcomes. For example, the response of winter wheat to cold temperature involves a complex series of events that begins with response to the stress imposed by the cold temperature, and then leads to cold acclimation and initiation of the vernalization process. Identification of phospholipid signaling in cold acclimation of winter wheat would provide heretofore unknown specific genes to target in molecular breeding efforts to manipulate cold acclimation rates and cold tolerance. An excellent example of the potential of this approach was shown by Welti et al. (2002), in which PLDα-deficient *Arabidopsis* plants (expressing antisense PLDα) survived exposure to -10ºC for 2hr, while identically-treated wild-type plants did not. This effect was attributed to a 23% reduction in degradation of phosphatidylcholine in the PLDα-deficient plants (Welti et al., 2002). Conversely, freezing tolerance was decreased by abrogation of PLDδ, and increased by its overexpression, indicating differing roles for different forms of PLD in the response to freezing temperatures (Li et al., 2004). Identification of genes with similar effects in the monocotyledonous wheat plant would provide a molecular basis to manipulate potentially the rate and extent of cold acclimation. It is not known whether

phospholipid signaling plays a role in this long-term process. It has been reported that the cold tolerance of winter wheat plants increased at genotype-dependent rates, with maximum or near maximum cold acclimation reached after four weeks of exposure to cold temperature (Fowler and Limin, 2004; Mahfoozi et al., 2001; Willemot, 1975). Hence, if phospholipid signaling is involved in the cold acclimation process, there should be evidence of the involvement throughout the four-week period, lasting much longer than the initial response to the onset of cold temperature.

The objectives of this study were to seek evidence of phospholipid signaling and to investigate the dynamics of phospholipid acyl side chain composition in the leaves of five winter wheat cultivars differing in cold tolerance of the acclimated plants, during five weeks of cold acclimation.

MATERIALS AND METHODS

The five winter wheat cultivars "El Tan,' Froid,' CDC Kestrel,' 'Oregon Feed Wheat #5,'and 'Tiber' were used. The LT_{50}, the temperature at which 50% of the plants were killed were determined as follows. Ten seeds from each population were planted in commercial soilless potting mix substrate in plastic cell packs, one seed per cell with randomization, and were grown at 22ºC under cool white fluorescent lights (200 µmol m^{-2} s^{-1}) in a growth chamber (Model E15, Conviron, Pembina, ND) with a 16-h photoperiod until the seedlings reached the three-leaf stage. Relative humidity was not controlled. The temperature was then lowered to 4°C for 20 days. Plants were then clipped 2.5 cm above the crown, the substrate was saturated with water, allowed to drain for one hour, and the flats were placed in a freezing chamber at 4ºC in darkness. During the artificial freeze test, the air temperature in the freezing chamber was reduced 1.5^{o}C/h to -25^{o}C (Fowler et al., 1981). One set of samples was removed from the freezing chamber when the soil temperature reached each of eight designated temperatures; -9, -11.5, -13, -14.5, -16, -17.5, -19, and -20.5^{o}C. After removal, the plants were transferred to a growth chamber as above and held for 24h at

4°C in darkness, then were transferred to the greenhouse. Plant regrowth was evaluated after 6 weeks (Storlie et al., 1998) and the proportion of plants to regrow of the ten planted in each flat comprised the survival score for that cultivar for that flat. The proportion of each cultivar that survived was determined at each of the eight temperatures tested. The entire experiment was replicated twice at separate times and LT_{50} scores were determined by regression analysis of the survival data on temperature to predict the temperature at which 50% of the plants would survive.

Phospholipid determination

Fifteen flats containing the five cultivars were planted with randomization of the cultivars within each flat. Plants were grown as above, and tissue was collected at weekly intervals for five weeks after the plants were transferred to 4°C. Each flat comprised a replication; tissue from three replications was collected each week. All growth above the soil line was harvested. Each sample consisted of at least 10 plants. Harvested tissue samples were immediately placed on ice, then were stored at -40°C until phospholipid extraction.

Phospholipids were extracted using the method described by Zabrouskov *et al.* (2001). The extraction solution was a 2:1 chloroform: methanol solution containing internal standard phospholipids (Avanti Polar Lipids, Alabaster, AL). Internal standards were phosphatidylserine$(14:0)_2$, phosphatidic acid $(12:0)_2$, phosphatidylglycerol $(14:0)_2$, phosphatidylcholine$(14:0)_2$, and phosphatidylethanolamine $(13:0)_2$ at 3.4 μg/μL, 5.7 μg/μL, 6.25 μg/μL, 15 μg/μL, and 1.75 μg/μL, respectively.

Electrospray ionizing mass spectrometry (ESI-MS)

Electrospray mass spectra were obtained using an API-4000 triple quadrupole mass spectrometer (MDS Sciex, Concord, Canada) using an Agilent I 100 (Palo

Alto, CA) system for autosampling and delivery of a bolus sample (5 μL) at a flow rate of 50 μL min^{-1} (methanol) and nebulized with the assistance of air. The mass spectrometer was operated at unit resolution (50% valley definition) for both Q1 and Q3 with Q2 operating in rf only mode with helium as collision gas (4 arbitrary units). The instrument was operated in positive ion mode for phosphatidyl- and lysophosphatidyl-choline, and phosphatidylserine, and in the negative ion mode for phosphatidic and lysophosphatidic acid, phosphatidyl- and lysophosphatidyl-ethanolamine, phosphatidyl- and lysophosphatidyl-glycerol, and phosphatidylinositol, with electrospray voltages of 4500 and -4000 V, respectively. All other mass spectral and fragmentation parameters (i.e., declustering potential, collision energy) were optimized individually for each lipid class using the standards described above to represent the lipid classes; phosphatidylglycerol $(14:0)_2$ was used to standardize all phosphatidylinositols. Quantitative analysis was performed in multiple reaction-monitoring mode (50 μs dwell time) observing the loss of the glycerylacyl portion (phosphatidylserine was observed with a potassium adduct) for 40 phospholipids (10 positive ion mode and 30 negative mode) and the respective internal standards for each class of known concentration. Quantification was made based on a standard curve developed from the lipid class standards. Comparisons were made throughout each lipid class by employing precursor ion scans of the resulting lipid head group. Each sample was probed three times, once for the precursor scan of each lipid class, and twice more for the quantitative analysis of the dominant lipids in positive and negative ion modes. Means and standard errors of these measurements were reported by the data collection and analysis software. The means of the two quantitation measurements were reported as the measured value of each phospholipid.

Phospholipid concentrations were determined on a ng/μL basis, and converted to molar values. All further comparisons of phospholipid occurrence were on a moles per total number of moles (molar percent) basis. Statistical means separations of phospholipid amounts were carried out with the PROC GLM

routine of the Statistical Analysis System (SAS Institute, 1999) using cultivar, weeks of acclimation, and replications as class variables. Means were separated with Duncan's means separation.

Quantitative real-time PCR

Messenger RNA transcript levels of five genes involved in phospholipid metabolism were measured after 0, 1, 2, 3, or 4 weeks of cold acclimation in winter wheat line 442. Line 442 is one of several near-isogenic lines that have been fully characterized with regard to their genetic constitution at the Vrn1-Fr1 locus, a major determinant of winter vs. spring growth habit, and cold tolerance, as measured by LT_{50} scores (Storlie et al., 1998). The reported average LT_{50} of 442 (-13.2ºC; Storlie et al., 1998) is close to the mean of the LT_{50} scores of the cultivars studied here (-13.3ºC, see Results). Therefore, it was thought that line 442 would represent typical gene transcript behavior in the winter wheats we studied.

The genes studied were phospholipase D, phospholipase C, phospholipase A_2, GDSL-motif lipase/hydrolase, and a lysophospholipase. The DNA sequence of phospholipase D was from GenBank accession number BE490064, whose amino acid sequence had 73% identity to *Arabidopsis* phospholipase Dζ. The sequence of phospholipase A_2 was from accession CA654135, 79% identical to barley PLA_2. The DNA sequence of phospholipase C was from TIGR (http://www.tigr.org/tdb/tgi/tagi/) accession number TC148099, identified by TIGR as 76% identical to phosphatidylglycerol specific PLC from *Arabidopsis*. The sequences of GDSL-motif lipase/hydrolase-like protein (TC143709), and lysophospholipase (TC171605) were also from TIGR. The Primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used to design primers consisting of about 20 nucleotides with melting temperatures around 60^{o}C, generating an amplicon of about 100 nucleotides. The primer sets were: phospholipase D, forward: 5' - AACCGTGTGTTGGAAGCAT and reverse: 5' - ATCAATGCCTCCCTGAAAAC; phospholipase C forward: 5' -

AACGAGACGGCTCTCATCAT and reverse: 5' – AAGGGTCAGGGCCAATAATC; phospholipase A2 forward: 5' – TAAGCTGCTTCTCCCGAATC and reverse: 5' - TACACCGACAGCTTCCCATT; GDSL-motif lipase/hydrolase-like protein forward: 5' - CCTACGGGTTCACGGAGTC and reverse: 5' - GAAGATGTGCTGGTCCCTGT; lysophospholipase forward: 5' - CTACCTGCTGCACAACCTGA and reverse: 5' - GTAGAGCTCCTGCGAGGCTA, respectively.

Trizol reagent (Invitrogen, San Diego, CA) was used to extract the total RNA. The whole leaves collected from each individual plant were ground in liquid nitrogen with a mortar and pestle. Two milliliters of Trizol solution was added to the mortar before thawing of the plant material, and total RNA was extracted following the manufacturer's instructions. Extracted total RNA was quantified with a spectrophotometer (Jasco V-530, Jasco Inc, MD, USA). The RNA quality was analyzed by running 2 µg of total RNA on a 1.2 % agarose gel. The total RNA was stored at – 80ºC. RNA extractions were performed on three replicate plants from each time point.

Quantitative RT-PCR was performed on a RotorGene 2000 unit (Corbett Research, Sydney, Australia) using SYBR green (product number S7567; Molecular Probes, Eugene, OR) for detecting the PCR products at the end of each amplification cycle. Superscript One-step RT-PCR Platinum Taq kit (Invitrogen, San Diego, CA) was used to construct the cDNA and carry out the PCR amplification in a single tube. The qRT-PCR profile consisted of constructing cDNA for 15 min at 50°C followed by a 3 min denaturation at 95°C, then 32 PCR cycles of 15 s at 95°C, 30 s at 54°C, and 30 s at 72°C. The qRT-PCR solution was composed of 1 X reaction buffer from the Superscript One-step RT-PCR kit, 1.7 mM $MgCl_2$, 1 µM primers, 1:42,000 SYBR Green, and 200 ng total RNA in 20 µL reactions, which were covered with 7 µL light mineral oil (Sigma, St. Louis, MO). Melting curves of the final qRT-PCR products were generated to confirm a single PCR product had been formed. Copy number

estimates were based on a standard curve drawn from the generated data from known concentrations of cloned wheat manganese-superoxide dismutase double-stranded DNA and primers described previously (Baek and Skinner, 2003). Standard errors of the measurements were determined with the built-in function in a Microsoft Excel spreadsheet.

RESULTS

The LT_{50} of each cultivar after cold acclimation was: 'El Tan' -13.6 ^{o}C, 'Froid' -15.7^{o}C, 'CDC Kestrel' -14.6 ^{o}C, 'Oregon Feed Wheat #5' -9.5 ^{o}C and 'Tiber' -13.5 ^{o}C.

The ESI-MS technique yielded highly reproducible quantification of the 40 phospholipid species identified in Table 1. This technique distinguishes phospholipid species by the total carbon and double bond content of the two fatty acid acyl side chains combined. A total of 22 acyl chain configurations were distinguished (Table 1).

Acyl chain dynamics of total phospholipids

The total phospholipid content of the leaves, as estimated by the sum of the 40 phospholipids measured (Table 1), increased significantly ($P<0.05$) in each of the cultivars during the first week of cold acclimation and remained significantly greater than initial levels throughout the five-week period (Fig. 1). 'Oregon Feed Wheat No. 5' had the least amount of cold tolerance (LT_{50}=-9.5 ^{o}C) and had significantly ($P<0.05$) less phospholipid than any of the other cultivars after acclimation (Fig. 1, means separation not shown). 'CDC Kestrel,' (LT_{50} = -14.6 ^{o}C), showed the greatest increase in phospholipid content (186% increase) and the greatest phospholipid concentration of the five cultivars after acclimation (Fig. 1).

The proportion of phospholipids with acyl groups consisting of 30 or 34 carbons, and hence consisting of mismatched acyl chain lengths, differed significantly among the cultivars at the start of cold acclimation, but the cultivars were statistically equal after one week and through the remaining four weeks (data not shown). The ratio of mismatched to equal side chains declined rapidly during the first week of cold acclimation, concomitant with a significant increase in total phospholipid concentration (Fig. 2). Total phospholipid content varied little or

decreased slightly in weeks 2-4, then increased again in the fifth week of cold acclimation (Fig. 2). The ratio of mismatched to equal side chains generally continued to decrease through the second week of cold acclimation, then increased through the fifth week (Fig. 2). The rebound of the mismatched:equal ratio largely was due to an increase in acyl group 34:1 (Fig 3).

Lysophospholipids (LPLs)

We monitored six lysophospholipids throughout the five-week cold acclimation period (Table 1). The lysophospholipids comprised a small portion of the total phospholipids, and most did not vary significantly between cultivars throughout the cold acclimation period. However, lysophophatidylcholine 18:2 and 18:3 occurred in statistically equal concentration in the five cultivars at the start of cold acclimation, but at the end of cold acclimation were found in significantly lower concentration in Oregon Feed Wheat #5 (the least cold-tolerant cultivar) than any other cultivar (Table 2). The quantity of lysophophatidylcholine 18:2 and 18:3 remained virtually unchanged during the first week of cold acclimation while total phospholipid content increased rapidly, but then increased over the remaining four weeks while total phospholipid content varied little (Fig. 4).

Phospholipase Transcriptional Dynamics

Of the five phospholipases examined, PLD, PLC, and PLA_2 appeared to be most responsive to cold exposure (Fig. 5). The mRNA expression level of PLD increased more than 3-fold, and of PLA_2 more than two-fold in the first week of cold exposure (Fig. 5). The transcript copy numbers of both PLD and PLA_2 had decreased by the end of the fourth week of cold acclimation, but remained higher than the initial levels (Fig. 5). In contrast, the initial PLC copy number was significantly greater than PLD or PLA_2 and the amount of increase was less, but the copy number continued to increase throughout the four-week period (Fig. 5). The expression of lysophospholipase and a GDSL-lipase were consistently

detected throughout the four-week period, but at low levels that varied little (Fig. 5).

DISCUSSION

We found that a rapid reduction in the proportion of mismatched acyl chains in wheat phospholipids occurred during the first week of cold acclimation, concomitant with an increase in total phospholipid content (Fig. 2). It has been shown that mismatched acyl chains in phospholipids comprising a typical bilayer membrane contribute to significant alterations in physical properties of the membrane (Ali et al., 1998; Fang et al., 2003) and associated nonphospholipid components (Maneri and Low, 1988; Pitotti et al., 1980; Volmer and Veltel, 1985). Taken together, these observations suggest that wheat membranes were re-engineered in response to cold temperature by the addition of phospholipids, most of which had acyl side chains of equal length (Fig. 2), probably impacting physical properties of the membrane itself, such as elasticity and tensile strength, and properties of membrane-associated components. As cold acclimation progressed, the proportion of mismatched acyl chains increased in all cultivars, and in two cultivars ('Kestrel' and 'Tiber') returned to the initial levels by the end of the five-week period studied, while total phospholipid content remained constant or increased only slightly (Fig. 2). Thus, it appeared that a response to cold included a rapid deposition of additional phospholipid, with equal-length side chains, followed by a restructuring of much of the newly added phospholipid to result in mismatched side chains.

Concomitant with the increase in total phospholipid content and the decrease in mismatched side chains, we observed an increase in activity (at the mRNA transcription level) of phospholipase D, phospholipase C, and phospholipase A_2. These enzymes are known to participate in phospholipid signaling, primarily membrane related signaling. The products of phospholipase A_2 include lysophospholipids; we observed a significant increase of two forms of lysophosphatidylcholine, which was related to cold tolerance in that the least cold tolerant cultivar accumulated significantly less lysophosphatidylcholine than any of the other cultivars tested. Lysophosphatidylcholine, well-known as a signal

molecule (Viehweger et al., 2002; Ryu, 2004), was reported to induce resistance to a fungal and a viral pathogen in Solanaceaous plants when exogenously applied (Spivak et al., 2003) and was shown to increase over a two-week period in response to phosphate starvation (Gniazdowska et al., 1999). We observed that the level of lysophosphatidylcholine changed very little in the first week of cold exposure, but then increased over the next four weeks of cold acclimation, resulting in more than 12-fold increase of the 18:3 form (Fig. 4). We suggest this pattern of increase may be indicative of involvement of this compound, and of phospholipase A_2-based signaling, in the cold acclimation/vernalization process that occurs after the initial response to cold stress.

CONCLUSIONS

We believe that the response of winter wheat to cold temperature includes a rapid augmentation of the phospholipid component of membranes, with a simultaneous increase in expression of phospholipases A_2, C, and D. Following the rapid augmentation, a structural re-engineering process occurs, resulting in a re-establishment of mismatched:matched acyl chain ratios and a decrease in transcriptional activity of phospholipase D and A_2. Expression of phospholipase C, at levels higher than any of the other phospholipases throughout these early events, continues to increase, suggesting involvement in downstream processes. The level of lysophosphatidylcholine, a molecule known to be involved in phospholipid signaling, also increased throughout the period studied.

We believe these observations are consistent with long-term phospholipid signaling playing a significant role in cold-temperature acclimation and later events initiated by exposure to cold temperature. While it generally is accepted that phospholipid signaling forms a vital part of a plant's rapid response to biotic and abiotic stress, responding in minutes, (Laxalt and Munnik, 2002), our results suggest phospholipid signaling also plays a continuing role in the weeks-long cold acclimation process. Wheat plants continue to grow during cold acclimation,

which requires four weeks or more to develop completely (Mahfoozi et al., 2001; Willemot, 1975; Fowler and Limin, 2004), and phospholipid signal molecules are of limited mobility (Wang et al., 2002). Therefore, it seems likely the processes indicated by phospholipid dynamics in this study are continued in new growth until the entire plant reaches maximum cold acclimation. We observed that lysophosphatidylcholine accumulation and phospholipase C expression continued to increase after four weeks of cold exposure, suggesting that phospholipid signaling continues to be involved in processes downstream of cold acclimation, perhaps vernalization.

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Figure Captions

Figure 1. Average amounts of total phospholipids in shoots of five winter wheat cultivars before and after five weeks of cold acclimation at 4^{o}C. Error bars indicate +/- one standard error unit.

Figure 2. The ratio of mismatched to equal acyl chain components and total phospholipid content in shoots of five winter wheat cultivars during five weeks of cold acclimation at 4^{o}C. All graphs are drawn to the same scale. The cultivars were: A - El Tan, B – Froid, C – CDC Kestrel, D – Oregon Feed Wheat #5, E – Tiber. Error bars indicate +/- one standard error unit.

Figure 3. Average changes in molar percent of phospholipid acyl chain pairs in five winter wheat cultivars during five weeks of cold acclimation at 4^{o}C. The first number indicates the total number of carbons comprising the acyl chain pair, the number after the colon indicates the total number of double bonds in the acyl chain pair.

Figure 4. Dynamics of total phospholipid content and of lysophosphatidyl choline 18:2 and 18:3 in winter wheat shoots during 5 weeks of cold acclimation. Values presented are the averages from cultivars El Tan, Froid, CDC Kestrel, and Tiber. Fold changes are indicated relative to initial values. Exposure to cold was initiated at 20 days of growth.

Figure 5. mRNA transcript number and corresponding fold-change in expression levels of five phospholipase genes in winter wheat accession 442 during four weeks of cold acclimation at 4^{o}C. All fold-change values are expressed relative to initial values, arbitrarily set to a value of one. The gene identification and the GenBank or TIGR (TC) sequences they were based on were: A – Phospholipase C, TC148099; B – Phospholipase D, BE490064; C – Phospholipase A_2,

CA654135; D – GDSL lipase/ hydrolase, TC143709; E – Lysophospholipase, TC171605. Error bars indicate +/- one standard error unit.

Table 1. Phospholipid species monitored throughout five weeks of cold acclimation of five winter wheat cultivars

	Total	
Phospholipid species	acyl carbons	double bonds
Phosphatidic acid	24	0
	36	2
	36	3
	36	4
	36	5
	36	6
	40	7
	40	8
	40	9
Phosphatidylserine	40	5
	40	4
	40	8
Phosphatidylinositol	28	0
	34	1
	34	2
	34	3
	36	4
	36	5
	36	6
Phosphatidylglycerol	34	2
	34	3
	34	4
	36	6
Phosphatidylethanolamine	34	2
	34	3
	36	4
	36	5
	36	6
	40	1

Phosphatidylcholine	34	2
	34	3
	36	3
	36	4
	36	5
Lysophosphatidylcholine	18	2
	18	3
Lysophosphatidylethanolamine	18	2
Lysophosphatidylglycerol	18	2
	18	4
Lysophosphatidic acid	22	0

Table 2. Lysophosphatidyl choline (LPC) accumulation in five winter wheat cultivars with indicated LT_{50} values after five weeks of cold acclimation.

		LPC 18:2†			LPC 18:3†		
		Day			Day		
Cultivar	LT_{50}‡	0	35	Fold-change	0	35	Fold-change
		nmol mg^{-1} fresh wt.	nmol mg^{-1} fresh wt.		nmol mg^{-1} fresh wt.	nmol mg^{-1} fresh wt.	
	ºC						
El Tan	-13.6	1.20a	6.42a	5.35	0.66a	6.21a	9.41
Froid	-15.7	1.14a	8.16a	7.16	0.60a	6.81a	11.35
Kestrel	-14.6	0.93a	6.30a	6.77	0.54a	5.70a	10.55
Oregon Feed Wheat #5	-9.5	0.99a	4.26b	4.30	0.84a	4.35b	5.18
Tiber	-13.5	0.87a	7.41a	8.52	0.42a	6.06a	14.43

† Values are the average of three trials. Values within a column followed by the same letter are not significantly different according to Duncan's multiple range test (P=0.05).

‡ LT_{50} is the temperature at which 50% of the fully-acclimated plants were killed.


2000
1800
1600
1400
1200
1000
800
600
400
200
0
Phospholipid content (nmol mg-1 fresh wt.)
Initial
5 weeks acclimation
El Tan
Froid
CDC Kestrel
Oregon Feed Wheat#5
Tiber
Cultivar

Figure 1.

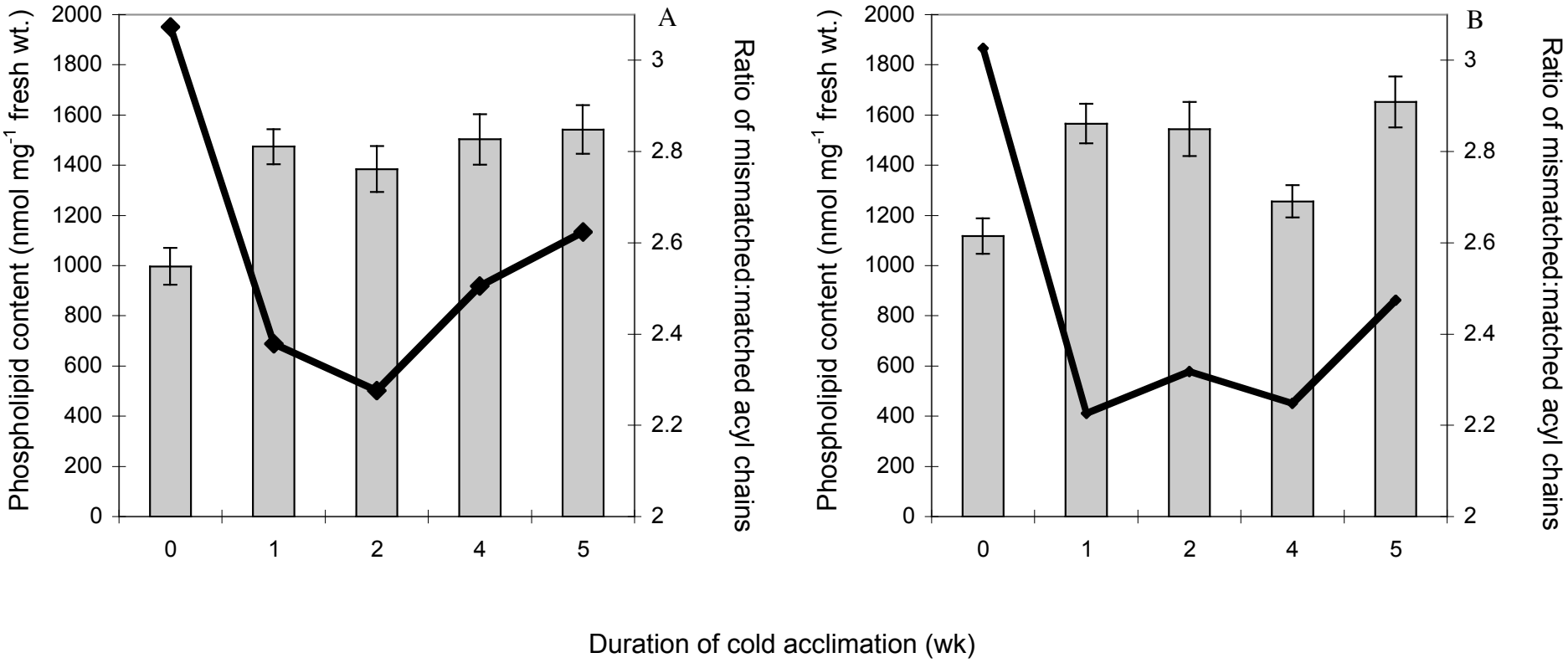
A
B
Phospholipid content (nmol mg-1 fresh wt.)
Ratio of mismatched:matched acyl chains
2000
1800
1600
1400
1200
1000
800
600
400
200
0
3
2.8
2.6
2.4
2.2
2
0
1
2
4
5
Duration of cold acclimation (wk)

Fig. 2 A,B

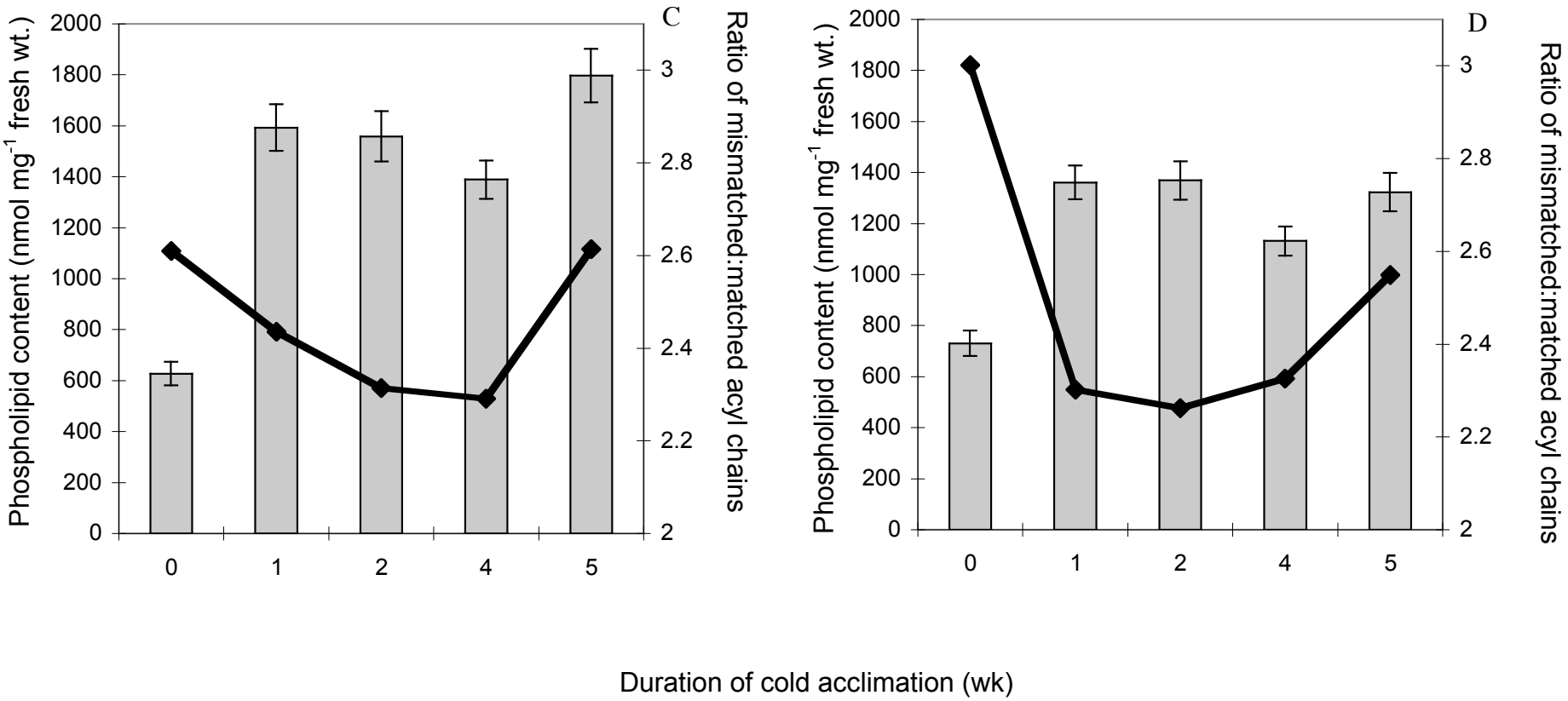
C
D
Phospholipid content (nmol mg-1 fresh wt.)
Ratio of mismatched:matched acyl chains
2000
1800
1600
1400
1200
1000
800
600
400
200
0
3
2.8
2.6
2.4
2.2
2
0
1
2
4
5
Duration of cold acclimation (wk)

Fig. 2 C,D

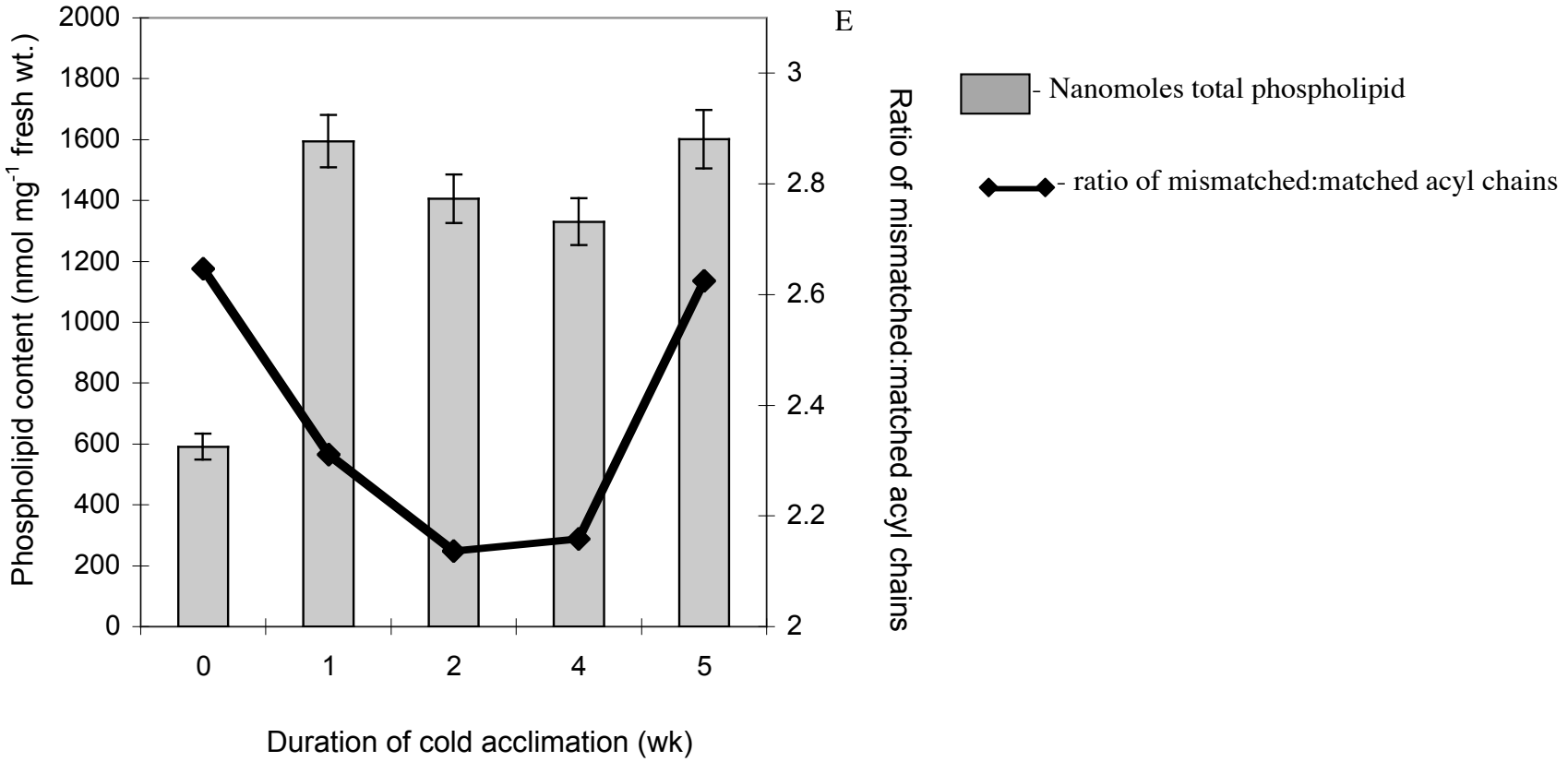
E
Phospholipid content (nmol mg-1 fresh wt.)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
3
2.8
2.6
2.4
2.2
2
Ratio of mismatched:matched acyl chains
0
1
2
4
5
Duration of cold acclimation (wk)
- Nanomoles total phospholipid
- ratio of mismatched:matched acyl chains

Fig. 2 E

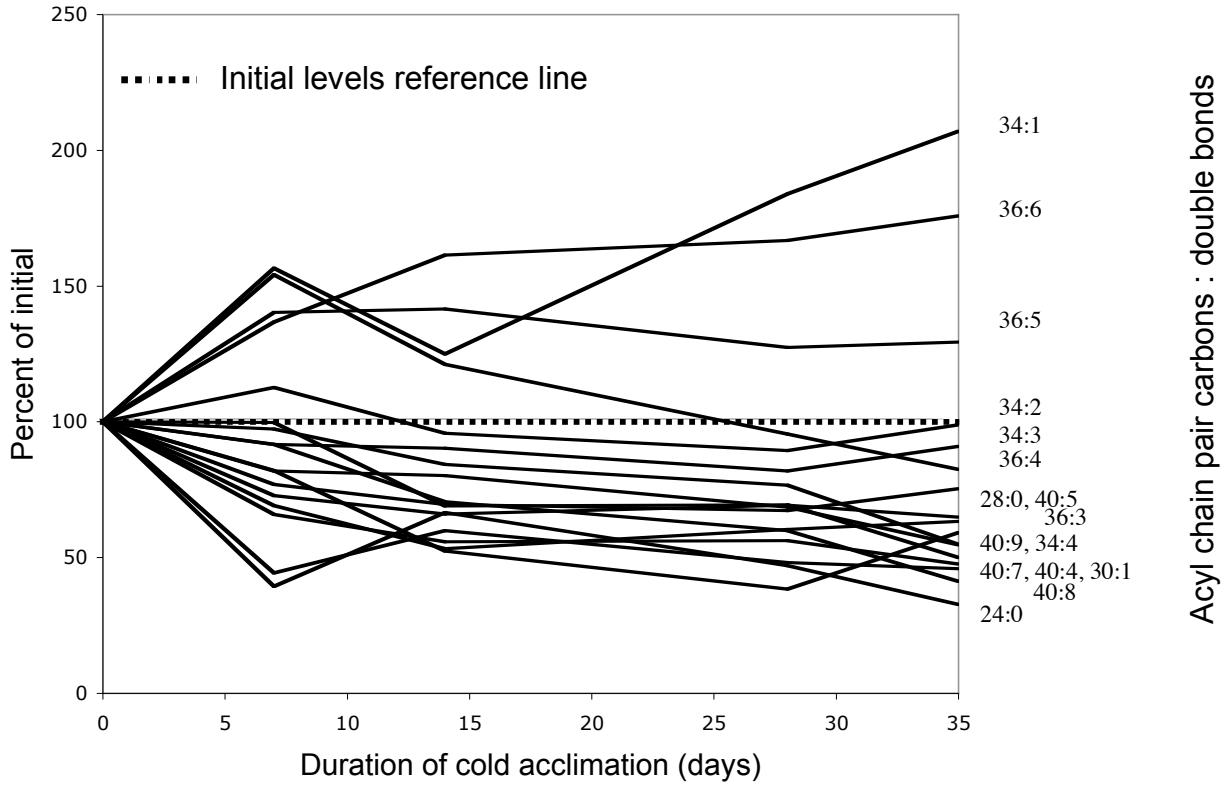
Initial levels reference line
Percent of initial
Duration of cold acclimation (days)
0
50
100
150
200
250
0
5
10
15
20
25
30
35
34:1
36:6
36:5
34:2
34:3
36:4
28:0, 40:5
36:3
40:9, 34:4
40:7, 40:4, 30:1
40:8
24:0
Acyl chain pair carbons : double bonds

Fig. 3.

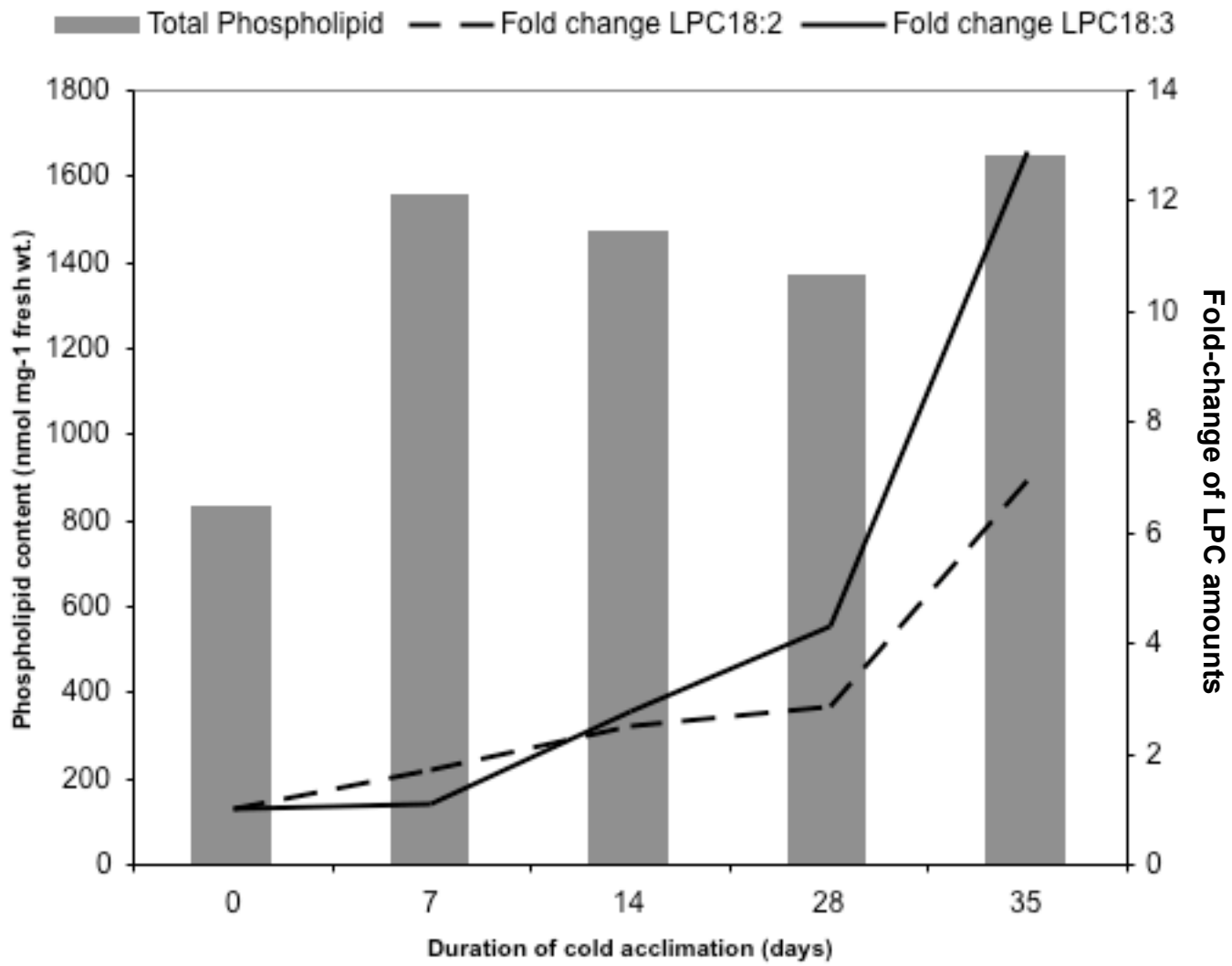
Total Phospholipid
Fold change LPC18:2
Fold change LPC18:3
Phospholipid content (nmol mg-1 fresh wt.)
Fold-change of LPC amounts
Duration of cold acclimation (days)
0
200
400
600
800
1000
1200
1400
1600
1800
0
2
4
6
8
10
12
14
0
7
14
28
35

Fig. 4.

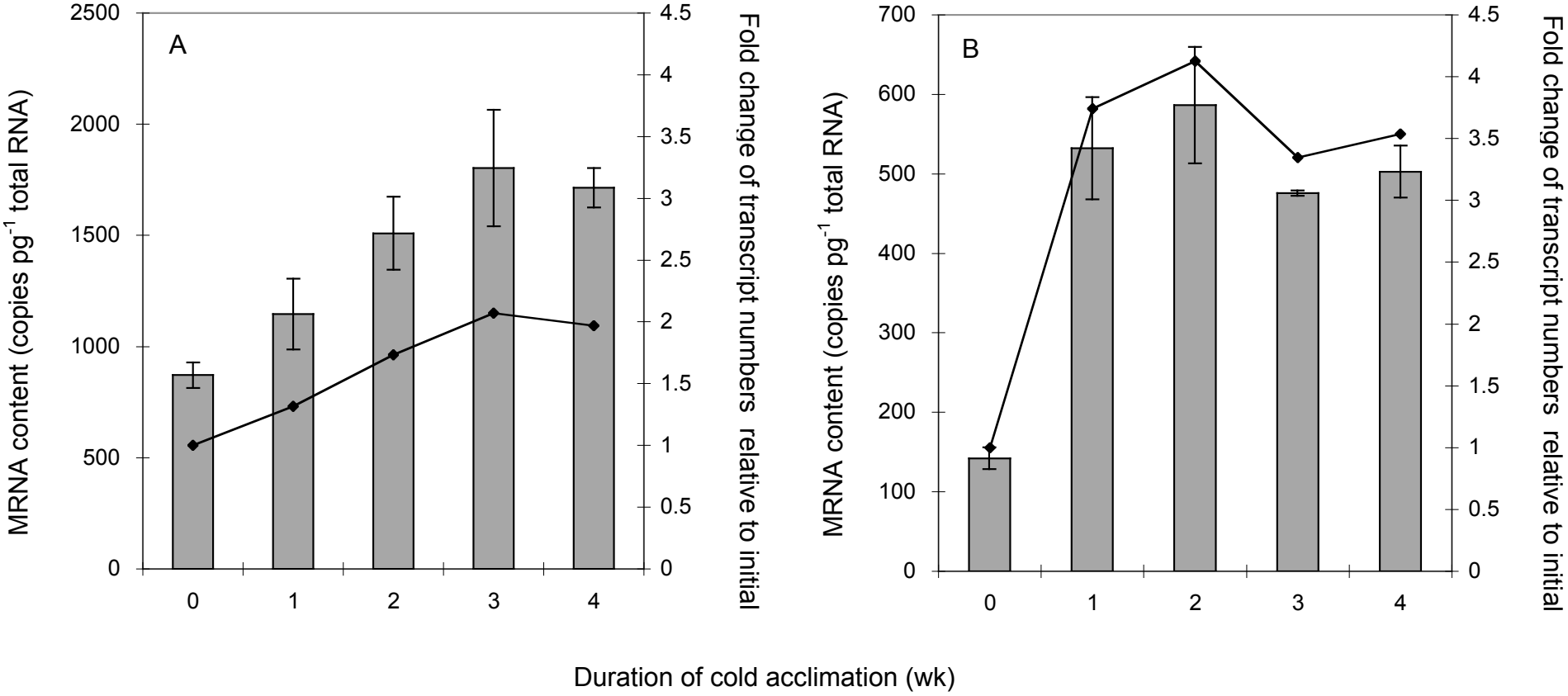
A
B
MRNA content (copies pg-1 total RNA)
Fold change of transcript numbers relative to initial
Duration of cold acclimation (wk)
0
500
1000
1500
2000
2500
100
200
300
400
600
700
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
1
2
3
4

Fig. 5 A, B

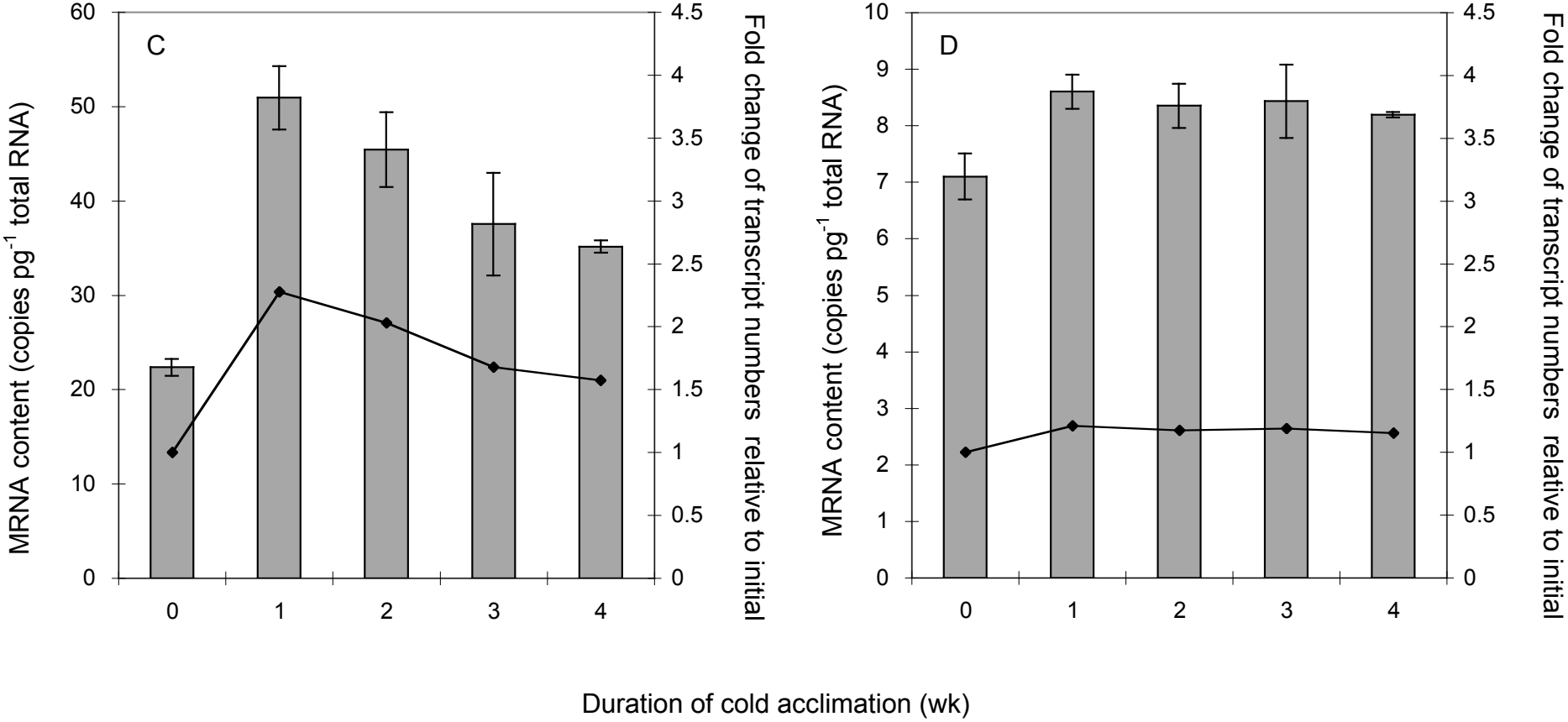
C
D
MRNA content (copies pg-1 total RNA)
Fold change of transcript numbers relative to initial
Duration of cold acclimation (wk)

Fig. 5 C, D

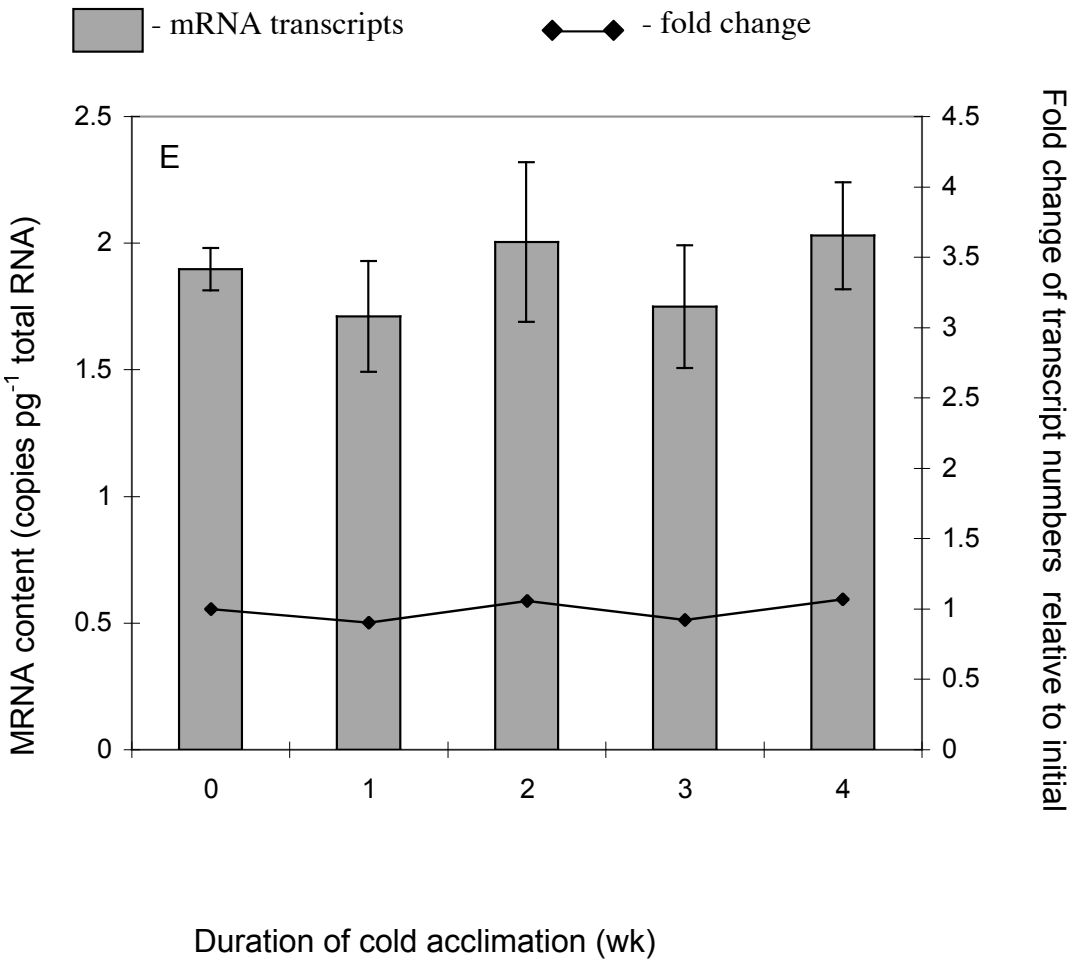
- mRNA transcripts
- fold change
E
MRNA content (copies pg-1 total RNA)
Fold change of transcript numbers relative to initial
Duration of cold acclimation (wk)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

Fig. 5 E